

Localized Characterization of an *In Vivo* Experimental Model of Post-Traumatic Osteoarthritis

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by

Nica de Nijs

Georgia Institute of Technology
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**LOCALIZED CHARACTERIZATION OF AN IN VIVO
EXPERIMENTAL MODEL AND POTENTIAL TREATMENT
METHOD OF POST-TRAUMATIC OSTEOARTHRITIS**

Approved by:

Dr. Robert Guldberg, Advisor
School of Mechanical Engineering
Georgia Institute of Technology

Dr. Nick Willett
School of Medicine
Emory University

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	iv
LIST OF TABLES	vi
LIST OF FIGURES	vii
LIST OF SYMBOLS AND ABBREVIATIONS	viii
SUMMARY	ix
<u>CHAPTER</u>	
1 INTRODUCTION	1
2 METHODOLOGY	3
Surgical Procedure	3
Gene Expression Analysis	3
Protein Expression Analysis	4
3 RESULTS	6
Articular Cartilage	6
Synovial Membrane	8
4 DISCUSSION	10
REFERENCES	12

LIST OF TABLES

	Page
Table 2.1: Genes studied in the microarray divided by the symptoms of OA that they are associated with in human OA.	5

LIST OF FIGURES

	Page
Figure 3.1: Clustergram of the gene expression of the medial cartilage.	6
Figure 3.2: Specific expression in the medial cartilage A. Osteopontin expression in Control, Sham, and MMT groups. B. Collagen 1 expression in Control, Sham, and MMT group. C. Collagen 10 expression in Control, Sham, and MMT group. D. Fibronectin expression in Control, Sham, and MMT group. E. MMP13 expression in Control, Sham, and MMT group.	7
Figure 3.3: Immunohistochemical stains of the cartilage with the top row coming from the week 3 sham group, and the bottom from the week 3 MMT group. Each column is a different stain: Collagen 2, Collagen 10, and MMP13 from left to right.	7
Figure 3.4: Clustergram of the gene expression in the medial synovial membrane.	8
Figure 3.5: Specific expression in the medial synovium A. MMP3 expression in Control, Sham, and MMT groups. B. MMP13 expression in Control, Sham, and MMT group. C. Collagen 1a1 expression in Control, Sham, and MMT group. D. Collagen 1a2 expression in Control, Sham, and MMT group. E. Osteopontin expression in Control, Sham, and MMT group.	8
Figure 3.6: Immunohistochemical stains of the cartilage with the top row coming from the week 3 sham group, and the bottom from the week 3 MMT group. Each column is a different stain: Collagen 1 on the left and MMP13 on the right.	9

LIST OF SYMBOLS AND ABBREVIATIONS

OA	Osteoarthritis
FDA	Food and Drug Administration
MMT	Medial meniscal transection
MMP	Matrix degradation proteins
μ -dHACM	Micronized, dehydrated amniotic membrane
EPIC- μ CT	Contrast enhanced micro-computed tomography
IHC	Immunohistochemical
RNA	Ribonucleic acid
cDNA	Combination deoxyribonucleic acid
ECM	Extracellular Matrix
DAB	3,3'-Diaminobenzidine
H&E	Hematoxylin and eosin
Saf-O	Safranin-O

SUMMARY

Osteoarthritis (OA) is a common chronic joint condition, affecting around 27 million people worldwide with an annual cost of over \$100 billion in the United States alone.¹ It is a degenerative disease classified by the progressive degradation of the articular cartilage due to processes such as depletion of proteoglycans, hypertrophic differentiation of chondrocytes, surface erosion, and lesion formation in the subchondral bone and cartilage.² Recently, OA has been linked to synovitis in the knee as well, showing that OA is a condition that effects the whole joint, not just the cartilage.³ The Medial Meniscal Transection (MMT) model is a post-traumatic mechanical model of OA, in which the destabilization of the medial meniscus results in characteristic features of human OA. Although the MMT model is the industry standard for therapeutic testing, localized expression events have not been characterized.⁴ To evaluate prospective tissue engineering and regenerative medicine approaches in OA, a reliable test bed must be established with well-characterized events.

In a microarray gene expression study, we have shown that, in the synovium and the articular cartilage, genes typically associated with OA showed similar altered expression in the MMT model. These results were then validated using immunohistochemistry tools. Sections of medial and lateral sides of the articular cartilage and synovium were stained for chondrogenic and osteogenic proteins, MMPs, and inflammatory cytokines, characterizing localized protein expression events in the model over time to trace the progression of artificial OA in the model and better establish a testing bed for regenerative medicine interventions for OA.

CHAPTER 1

INTRODUCTION

OA used to be considered a purely mechanical cartilage-degrading disease. Studies mostly concentrated on establishing an animal model that closely mimicked the degradation and altered mechanics of the cartilage.⁵ Studies of this kind relied on the assumption that OA is mostly caused by abnormal mechanical stresses and strains on the knee. This assumption has not only been proven incorrect, but also made it difficult to catch the disease before major damage had been done to the knee. When the lesions were big enough to be noticed and OA diagnosed, only a total joint replacement could have any effect in alleviating symptoms.⁶

The fact that OA affects cartilage and subchondral bone has been well established.⁷ However, not all symptoms of OA can be completely explained by defects in the cartilage and bone. OA is often associated with symptoms of inflammation such as joint pain, swelling, and stiffness. These symptoms are caused by synovitis in the joint based on the infiltration of activated B cells and T lymphocytes and the overexpression of pro-inflammatory mediators.³ This helps better understand OA, as it shows that it is a full joint condition, affecting the synovium, cartilage, and subchondral bone.

A study from the Guldberg lab used a micronized, dehydrated amniotic membrane (μ -dHACM) known as AmnioFix that was a decellularized extracellular matrix treatment method to deliver a large quantity of growth factors to the targeted area.² This attempted to attenuate cartilage degradation in the MMT rat model of OA. Animals were divided into three groups: the first received the AmnioFix injection without an MMT surgery, the

second received the surgery and the injection, and the third received the surgery but only a saline injection. Of the two groups that received the MMT surgery, the ones who had also received the injection showed no lesions and significantly reduced partial erosions. This shows that AmnioFix does have a protective effect on the lesion formation in the MMT model. AmnioFix has a range of immunomodulatory factors that may ameliorate OA progression. However, one shortcoming of an extracellular matrix type treatment method is that it is difficult to know exactly why it is doing what it is doing. Further studies are necessary to figure out exactly which factors of OA and what specific pathways are affected by the treatment.

Our study aims to investigate localized disease-modifying mechanisms involved in the disease progression of the MMT model. First, a microarray was used to study gene expression in the cartilage of the medial tibial plateau and the medial synovial membrane. Then, fluorescent immunohistochemical staining was used to confirm these results by studying protein expression throughout the joint space as well. Identified changes in gene expression could be used to confirm the similarities between human OA and the progression of the MMT model.

CHAPTER 2

METHODOLOGY

Surgical Procedure

Surgeries were done on the left legs of Lewis rats only, allowing the right legs to be used as contralateral controls. The animals were divided into groups of 9, depending on if they were to receive the MMT or sham surgeries. In each surgery, a small incision was made on the medial side of the left knee. The medial collateral ligament was exposed by blunt dissection and transected to visualize the joint space. For the MMT rats, the medial meniscus was transected completely at its narrowest point. Sham surgeries were used as another control to show that the surgery itself is not causing some of the expression changes until the meniscus is transected so for the sham rats, the meniscus was not cut. After this, the incisions were closed using staples and casting. After one week, an equal number of animals from each group were euthanized. For the other rats, the staples were removed. Three weeks after surgery, the remaining rats were euthanized. In all cases, after euthanasia, samples from the medial and lateral tibial cartilage and synovium were taken from all legs for further study.

Gene Expression Analysis

Samples were taken from the medial side of the articular cartilage on the tibial plateau as well as the medial synovium. RNA was extracted from the samples and, from that, cDNA was isolated. This cDNA was used in a Fluidigm microarray of multiple

different genes that have been shown to be affected in human OA. The genes tested are listed in **Table 2.1**. The results of this study were expressed in clustergrams comparing the overall gene expression as well as plotting the change in expression in each gene individually.

Symptom of OA	Genes Studied
ECM degradation	MMP3, MMP9, MMP13, Timp1, ADAMTS4, ADAMTS5
Chondrocyte hypertrophy	BMP2, Osteocalcin, Osteoprotegerin, Osteopontin, Frizzled-related protein, Grem1, Sox9
Cell apoptosis	Casp8, Tgm2
Inflammation	TNF α , TGF β -1, IL1-ra, IL1b, IL6, IL8, IL10, IL17, CCL2, CCL3

Table 2.1 Genes studied in the microarray divided by the symptoms of OA that they are associated with in human OA.

Protein Expression Analysis

Full joints were collected from the animals following euthanasia. These were then decalcified using Immunocal, dehydrated, and embedded in paraffin. The entire sample was then sectioned manually by cutting 5 μ m sections using the microtome. These sections were then used for immunohistochemical staining.

Sections from the edge and center of the medial condyle were stained using immunohistochemistry (IHC). Sections were first deparafinized and rehydrated using heat, xylene, pure ethanol, and 95% ethanol followed by a wash in tap water. The sections then went through antigen retrieval, where they were suspended in a sodium citrate buffer in a 60 °C hot water bath overnight. Following this, slides were treated with Triton X before being encircled by a hydrophobic barrier drawn on using a PAP pen.

At this point, the samples were ready to be treated with the primary antibodies that were left on the slides overnight in a humidity chamber at 20 °C. The next day,

sections were treated with a normal anti-goat serum followed by a fluorescent secondary antibody. After an hour, DAPI was added to the sections and they were sealed with a coverslip. Images of these sections were taken with a confocal microscope.

CHAPTER 3

RESULTS

Articular Cartilage

The microarray showed differences in a variety of gene expression in the medial cartilage. The clustergram (**Figure 3.1**) showed that the MMT surgeries clustered on one side of the chart and the sham and control surgeries on the other side, implying that the sham and control groups were more like each other and the MMT was different.

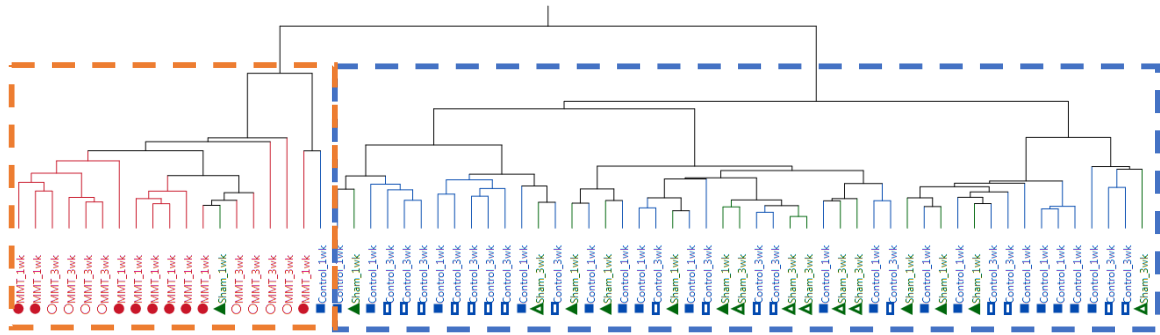


Figure 3.1 Clustergram of the gene expression of the medial cartilage.

More specifically, **Figure 3.2** shows the relative expression of five of the genes studied. Chondrogenic hypertrophic genes osteopontin and collagen 1 showed similar expression in the sham and control groups with increased expression in the MMT group. The same was true for ECM protein fibronectin and matrix degradation protein MMP13. For collagen 10, the sham and control groups showed similar expression and decreased expression in the MMT group.

The IHC stains of the cartilage, shown in **Figure 3.3**, also showed the differences between the MMT group and the control groups. Stains for collagen 1, collagen 2,

collagen 10, osteopontin, MMP3, and MMP13 could be seen prevalently in the images from the MMT group while the images from the sham group primarily showed the DAPI counterstain.

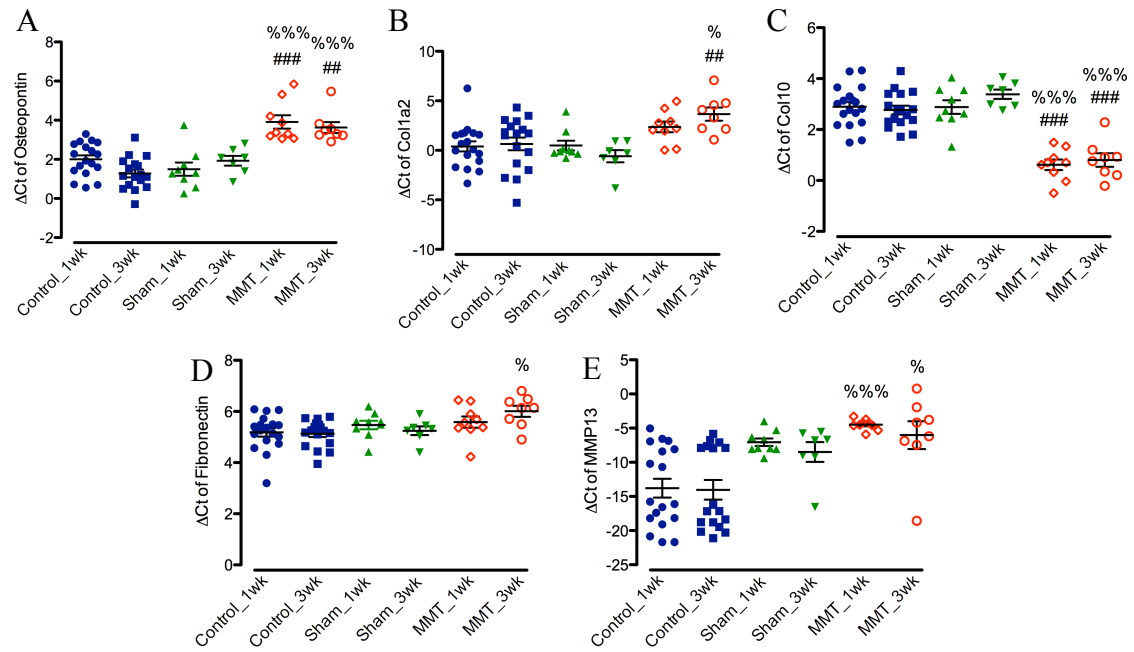


Figure 3.2 Specific expression in the medial cartilage A. Osteopontin expression in Control, Sham, and MMT groups. B. Collagen 1 expression in Control, Sham, and MMT group. C. Collagen 10 expression in Control, Sham, and MMT group. D. Fibronectin expression in Control, Sham, and MMT group. E. MMP13 expression in Control, Sham, and MMT group.

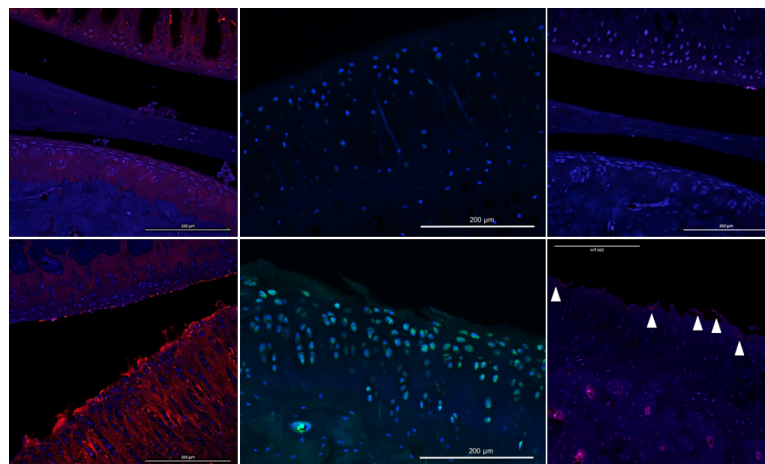


Figure 3.3 Immunohistochemical stains of the cartilage with the top row coming from the week 3 sham group, and the bottom from the week 3 MMT group. Each column is a different stain: Collagen 1, Collagen 2, Collagen 10, and MMP13 from left to right.

Synovial Membrane

In the medial synovial membrane, the clustergram (**Figure 3.4**) showed that the MMT group was clustered with the week 1 sham samples while the week 3 sham samples and control group were clustered on the other side.

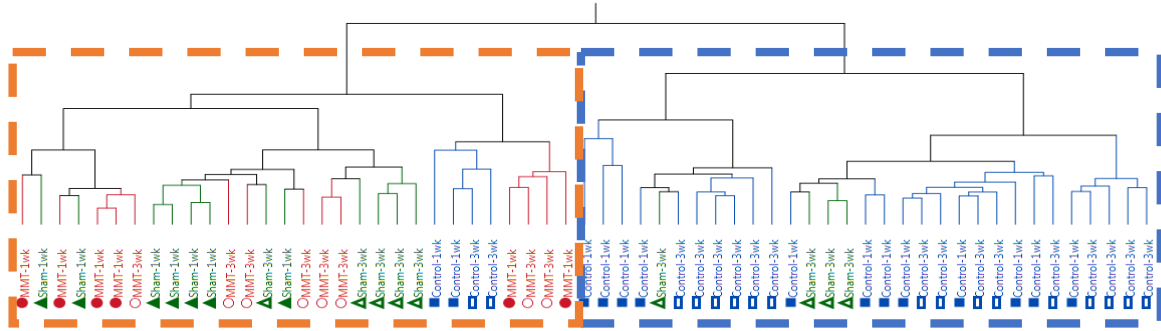


Figure 3.4 Clustergram of the gene expression in the medial synovial membrane.

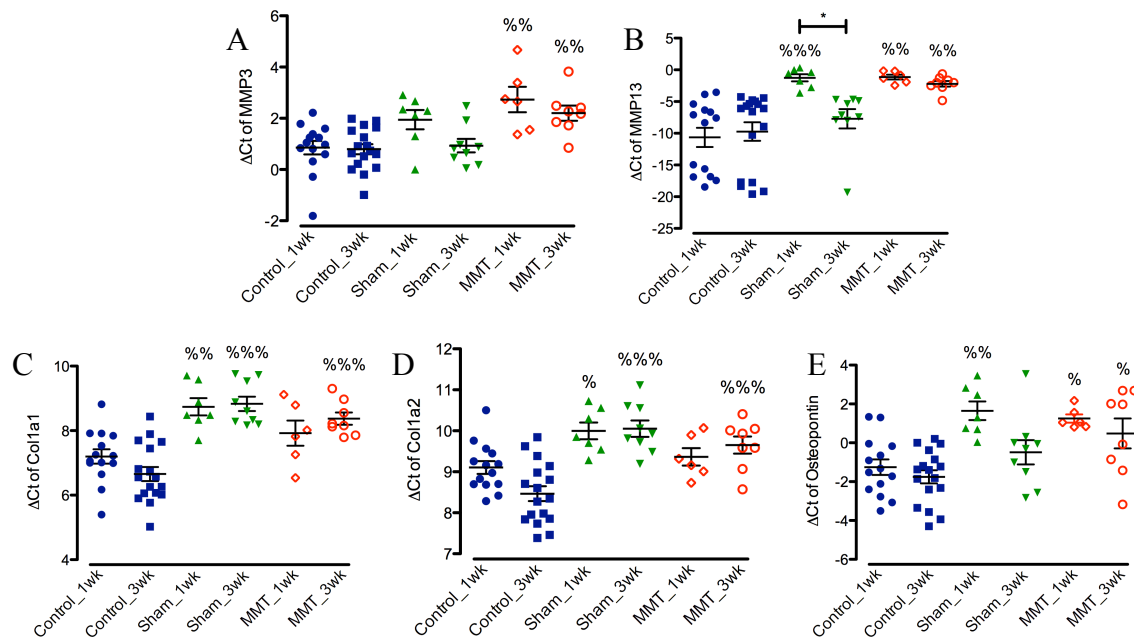


Figure 3.5 Specific expression in the medial synovium A. MMP3 expression in Control, Sham, and MMT groups. B. MMP13 expression in Control, Sham, and MMT group. C. Collagen 1a1 expression in Control, Sham, and MMT group. D. Collagen 1a2 expression in Control, Sham, and MMT group. E. Osteopontin expression in Control, Sham, and MMT group.

Specifically, **Figure 3.5** shows the relative gene expression of five genes studied in analyzing the synovial membrane. For MMP3, the sham and control groups showed

similar expression while it was upregulated in the MMT groups. For MMP13 and Osteopontin, the control and the week 3 sham group showed similar expression while the week 1 sham group and MMT groups showed overexpression. For collagen 1a1 and collagen 1a2 the sham and MMT groups were significantly upregulated over the control groups.

The IHC Stains of full joint samples backed up the findings in the gene expression study. As shown in **Figure 3.6**, there were differences in the prevalence of collagen 1, osteopontin, MMP3, and MMP13 between the MMT samples and the sham samples.

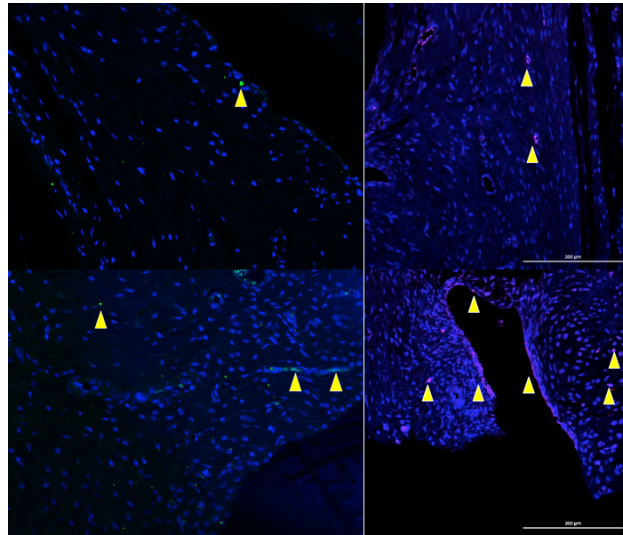


Figure 3.6 Immunohistochemical stains of the cartilage with the top row coming from the week 3 sham group, and the bottom from the week 3 MMT group. Each column is a different stain: Collagen 1 on the left and MMP13 on the right.

CHAPTER 4

DISCUSSION

OA causes breakdown of the extracellular matrix, chondrocyte hypertrophy, cellular apoptosis, and inflammation of the synovial membrane, all of which are caused by changes in gene and protein expression within the cartilage and, to a certain extent, in the rest of the joint. In humans, the genes associated with these factors have been well classified. We hypothesized that the MMT model simulates those same changes to an extent and is therefore a valid model of preclinical OA.

The results of the microarray showed similar upregulation in approximately 70 percent of the genes studied, implying that most of the same inflammatory, degradation, hypertrophy, and apoptosis pathways that are part of human OA are also part of the mechanisms of the MMT model. The effect on gene expression was related to pathways in chondrocyte hypertrophic, extracellular matrix (ECM) composition and degradation, and cartilage catabolism.

The protein expression results confirmed the results of gene expression study. Specifically, the Collagen 2 stain shows the morphological effects of the model on the cartilage, including fibrillations on the cartilage and a lesion beginning to form, as compared to the sham which the cartilage remained smooth. The changes expressed in the MMT model mirror those typically seen in OA. For the other stains, all showed greater expression of the proteins in the MMT model than the sham, implying changes in the protein expression and backing the findings of the microarray.

Altogether, the gene and protein analysis of specific locations in the joint space show that the MMT model is a good representation of OA. The gene expression analysis showed that there were similar genetic changes in the model to those seen in various other studies of human OA. The protein expression study confirmed these results as well as the local morphological changes of the model. With more extensive studies to fully characterize the gene and protein expression profiles of the MMT model, the model could become useful as a test bed for tissue engineering and regenerative medicine approaches to OA.

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